Immunity, Vol. 5, 505–511, December, 1996, Copyright ©1996 by Cell Press

Receptor Editing, Immune Diversification, and Self-Tolerance

Review

Marko Z. Radic* and Moncef Zouali†
*Department of Microbiology and Immunology
Allegheny University of the Health Sciences
Philadelphia, Pennsylvania 19129
†Département d'Immunologie
Institut Pasteur
28, rue du Dr Roux
75015 Paris
France

Over the last few years, our knowledge of the mechanisms that control the development of B cells and shape their repertoire has expanded rapidly, with description of a number of novel cellular and molecular pathways. Among these, receptor editing is unique because it enables B cells to alter their immunoglobulin variable (V) region genes and, consequently, change the specificity of the B cell receptor (BCR) expressed at their surface. This remarkable property provides clues to long-standing questions pertaining to the fate of cells developing in the bone marrow. The majority of B lymphocytes that arise in the bone marrow do not migrate to peripheral organs to participate in an immune response but, instead, undergo apoptosis and are disposed of by resident macrophages (Osmond et al., 1994). Two reasons for the death of immature B cells are the nonproductive assembly of the heavy (H) or light (L) chain gene segments and the generation of BCR that react against self. A third, related reason is that B cells may undergo apoptosis after they have exhausted their potential for additional V gene rearrangements. The displacement of productive rearrangements by additional, productive rearrangements on the same allele may be accomplished by secondary rearrangement at the H chain locus resulting in H chain replacement, or by secondary L chain V gene rearrangements, resulting in L chain editing. Thus, receptor editing depends on the capacity of variable light (VL) and heavy (VH) genes to undergo successive rearrangements. It may provide an alternative to apoptosis of immature B lymphocytes in the bone marrow if it occurs during the 16–18 hr period after cross-linking of the BCR, but before irreversible commitment to apoptosis (Rothstein, 1996). As will be discussed here, secondary rearrangements have now been described in a number of systems. Although formal proof that they are triggered by antigen encounter is lacking, mounting evidence suggests that some secondary rearrangements are a result of receptor engagement. Receptor editing not only diversifies the repertoire, but also may allow B cells to avoid high affinity autorecognition. Hence, receptor editing has implications with regard to maintenance of B cell tolerance and, possibly, to the induction of pathogenic autoimmunity.

Receptor Editing and Organization of the Immunoglobulin Variable Gene Loci

Functional immunoglobulin and T cell receptor genes are assembled by VDJ recombination, a process targeted by sequence motifs flanking the germline segments. Each motif, termed recombination signal sequence (RSS), is composed of conserved 7 (heptamer) and 9 bp (nonamer) motifs separated by a spacer of conserved length (12 or 23 bp) but of relatively variable sequence. Typically, recombination occurs between two RSS, each having a different spacer length (Lewis, 1994). Primary rearrangements at the κ chain locus are well suited for further rearrangements because rearranged $V_\kappa J_\kappa$ gene segments usually are flanked by unrearranged V_κ and J_κ segments (Figure 1A). Analysis of B-cell tumor models established that rearranged κ chain alleles can be replaced by secondary rearrangements of upstream V_κ genes to downstream, unrearranged J_κ gene segments (Selsing et al., 1984; Feddersen and Van Ness, 1985; Shapiro and Weigert, 1987; Levy et al., 1989; Lewis, 1994). Continuous secondary rearrangements of V_κ genes have also been observed in Abelson murine leukemia virus (AMuLV)-transformed pre-B cell lines (Lewis et al., 1982). Depending on the orientation of the editing V_κ relative to the J_κ, the secondary rearrangement may delete the primary $V_\kappa J_\kappa$ or separate it from C_κ by inversion. The remains of the primary, productive rearrangements can be recovered as extrachromosomal DNA circles produced by a secondary deletional rearrangement (Harada and Yamagishi, 1991) or, following an inversion, upstream on the same chromosome as the new $V_\kappa J_\kappa$ (Feddersen and Van Ness, 1985; Huber et al., 1992).

For the H chain, VHDJH rearrangements remove all available, unrearranged D gene segments on the same allele. Moreover, secondary VH rearrangements to downstream JH gene segments are not compatible with the 12-23 spacer rule that governs the joining of RSS. However, early studies of VH gene rearrangements in fetal liver–derived AMuLV-transformed pre-B cell lines prompted the search for an alternative mechanism of repertoire diversification. The observation that the JH-proximal VH81X gene dominates the repertoire in early ontogeny led to the suggestion that a primary rearrangement may serve as a substrate for subsequent VH gene replacement (Yancopoulos et al., 1984). Working independently, two groups soon provided evidence for a novel type of rearrangement between a RSS 3′ of an upstream VH gene and a heptamer sequence embedded within a rearranged VH gene (Kleinfield et al., 1986; Reth et al., 1986). This heptamer is found in the third framework region (FR3) of over 70% of murine VH genes and is identical to the heptamer signal sequences of D segments (Figure 1B). Invasion of an existing VHDJH rearrangement by a VH gene can repair a nonproductive VHDJH junction (Reth et al., 1986), as well as replace a functional H chain (Kleinfield et al., 1986; Covey et al., 1990).

Collectively, these studies suggested that secondary V gene rearrangements can be readily observed and that they take place even when the initial rearrangement is productive. H chain replacement is difficult to document in vivo because no more than a few base pairs of the original VH remain behind after receptor editing.

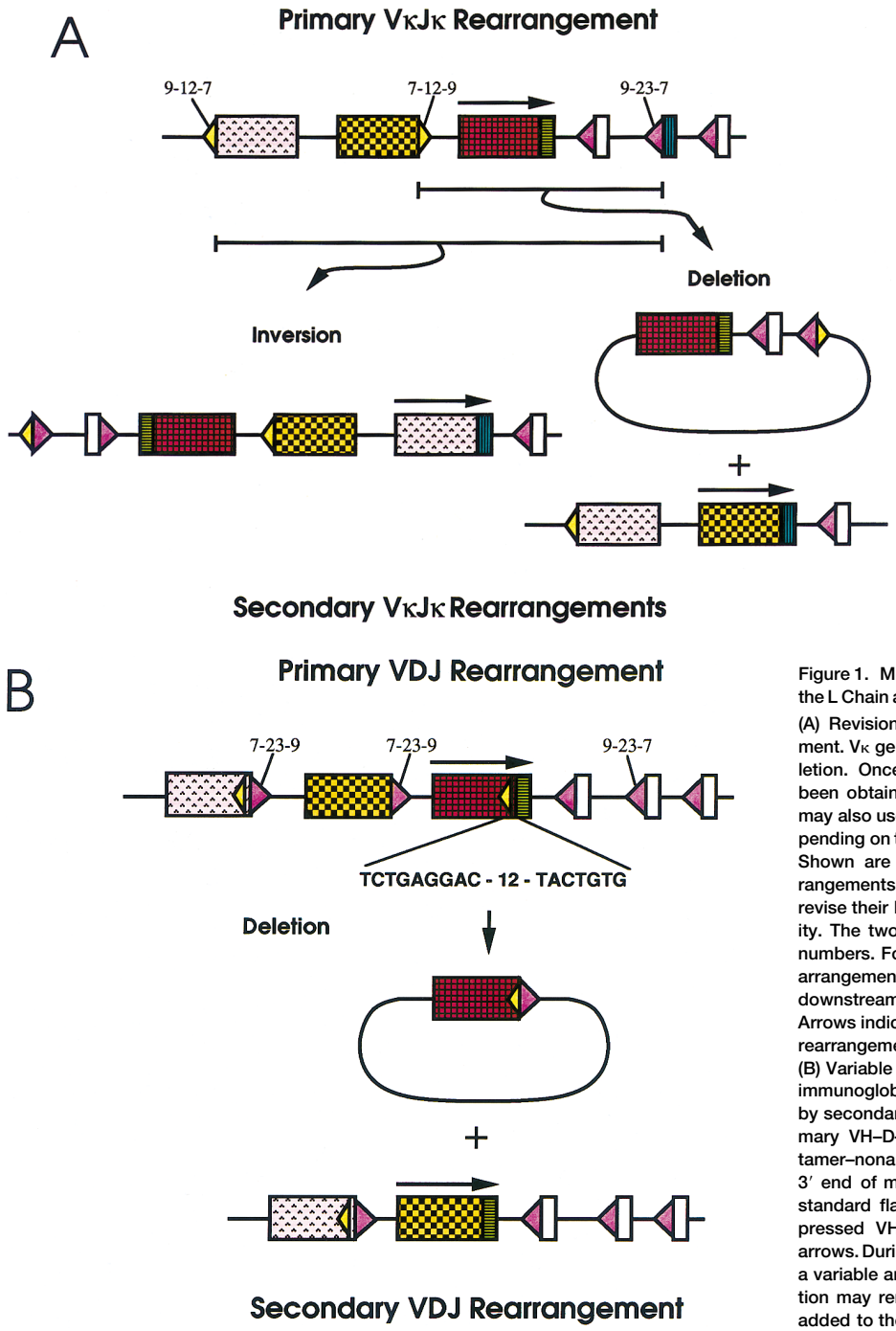

Figure 1. Mechanisms of Receptor Editing at the L Chain and H Chain Immunoglobulin Loci

(A) Revision of a primary Vκ–Jκ rearrangement. Vκ genes rearrange by inversion or deletion. Once a primary rearrangement has been obtained, a secondary rearrangement may also use either deletion or inversion, depending on the orientation of the Vκ involved. Shown are both types of secondary rearrangements that may be used by B cells to revise their BCR and acquire a new specificity. The two different RSS are indicated by numbers. For simplicity, both secondary rearrangements are shown to involve the same downstream Jκ, but different Vκ genes. Arrows indicate each of the expressed Vκ–Jκ rearrangements.

(B) Variable gene replacement at the H chain immunoglobulin locus. Editing of a VH gene by secondary rearrangement deletes the primary VH–D–JH joint. The conserved heptamer–nonamer that is embedded within the 3′ end of most VH germline genes and the standard flanking RSS are shown. The expressed VH–D–JH joints are indicated by arrows. During the secondary rearrangement, a variable amount of the original VH–D junction may remain and N nucleotides may be added to the new junction.

Nevertheless, circular DNA containing the edited VH gene joined precisely to the RSS of the new VH can be obtained (Usuda et al., 1992). The frequency of these secondary molecular events during normal B cell development remains unknown.

Editing of Autoreactive B Cells

Direct insights into receptor editing came from experiments in mice expressing autoreactive immunoglobulin transgenes that induce B cell deletion. In one model system, receptor editing was explored in mice expressing transgenes encoding an anti-H-2K^k/H-2K^b immunoglobulin M (IgM)/κ BCR (Tiegs et al., 1993). Transgene-encoded immunoglobulin were readily expressed in H-2K^d mice, but could not be detected in the periphery of mice expressing the K^k or K^b antigens. Idiotype-positive, immature B cells from the bone marrow of such mice expressed elevated levels of recombination-activating genes (*RAG-1 and RAG-2*), generated increased amounts of circular gene excision products, and yielded higher frequencies of endogenous λ chains in peripheral

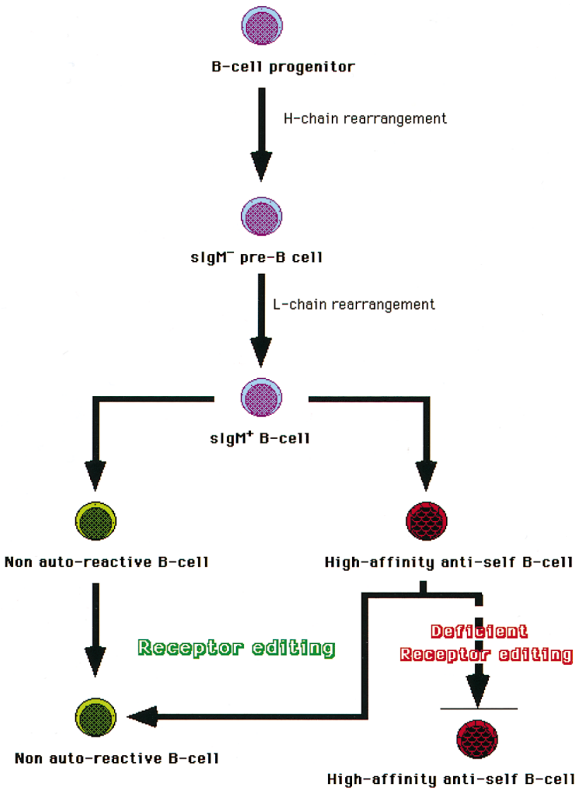

Figure 2. Extinction of Self-Reactivity by Receptor Editing

The immunoglobulin gene recombination machinery may give rise to a sIgM$^+$ B cell with self-specificity. To avoid autorecognition, this immature B cell may extinguish its autorecognition by revising its BCR through receptor editing. If, as might be the case in systemic autoimmune diseases, this mechanism is deficient, the B cell will remain autoreactive.

B cells (Tiegs et al., 1993). The increased L chain rearrangements led to the displacement of the transgene-encoded L chains by endogenous L chains and resulted in BCR with edited specificities that no longer elicited B cell deletion. This observation, together with the displacement of the transgene-encoded L chain by endogenous L chains in anti–DNA H and L chain transgenic mice advanced the concept that one form of receptor editing may utilize competition between two L chains for association with the transgene-encoded H chain (Gay et al., 1993; Tiegs et al., 1993). If the endogenous L chain competes effectively for the H chain and prevents binding to self-antigen, this form of receptor editing is able to avert B cell deletion (Figure 2).

The first detailed analysis of L chain editing in the absence of a L chain transgene became feasible by using an anti–DNA H chain transgene, 3H9, that imposes double-stranded (ds) DNA binding on a wide range of heterologous L chains (Radic et al., 1991). In 3H9 H-chain only mice, the absence of dsDNA-specific B cells indicates their efficient deletion (Erikson et al., 1991). Conversely, genetic analysis of the remaining B cells provides insights into receptor editing as it operates on authentic L chain substrates. Editing of anti–dsDNA BCR by secondary VκJκ rearrangements that avoid dsDNA binding greatly reduces the diversity of the observed Vκ repertoire in the periphery and biases the Jκ use toward the last available Jκ segment, Jκ5 (Radic et al., 1993a). No rearrangements of Vλ to Jλ were seen because combinations between 3H9 and Vλ1 or Vλ2 give rise to dsDNA-binding BCR and B cell deletion.

Transgenes incorporating a CDR2 arginine substitution in 3H9 (56R) that raises the antibody affinity for dsDNA 10-fold (Radic et al., 1993b) further reduced the expressed Vκ repertoire and increased the bias toward Jκ5 (Chen et al., 1994). In addition, 56R transgenic B cells displayed a novel strategy, the deletion of the entire H chain transgene array, which allowed them to express endogenous H chains and sidestep tolerance. To tilt the balance even more in favor of dsDNA binding, the 76R line of transgenic mice was constructed by incorporating a second arginine substitution that raises the dsDNA affinity of the 3H9 H chain by two orders of magnitude (Radic et al., 1993b). All three H-chain only transgenic mouse lines, 3H9, 56R and 76R, were backcrossed to JH deletion homozygotes to prevent the expression of endogenous H chains (Chen et al., 1994). In these mice, the transgenes allowed the development of B cells beyond the B220$^+$, CD43$^+$ pro-B cell stage at which lymphocytes of transgene negative JH$^{-/-}$ littermates were arrested. However, 56R, and especially 76R, transgenes impaired further lymphopoiesis at the immature B cell stage, and few if any IgM$^+$ cells were observed to develop. This blockade identified the transition from pre-B to surface Ig$^+$ (sIg$^+$) immature B cells as the stage at which anti–dsDNA B cell deletion occurs (Chen et al., 1995a).

A direct evaluation of receptor editing of H or L chain genes in their proper genomic context became possible by inserting functional V gene rearrangements at the Jκ (Prak and Weigert, 1995) or JH loci through homologous recombination in embryonic stem cell lines. This approach demonstrated that an engineered locus containing the 3H9 rearrangement effectively establishes allelic exclusion, participates in an immune response to a foreign antigen, undergoes isotype switching, and accumulates somatic mutations (Chen et al., 1995b). Most importantly, analysis of these "knock-in" mice confirms that H chain replacement occurs at the endogenous locus, that it can recruit distant VH genes, and that it can prevent dsDNA binding. Moreover, the editing mechanism generates imprecise joints between the new VH and its target, such that either a few base pairs of the original VH remain or part of the original CDR3 is deleted and nontemplated bases are added at the new junction. Interestingly, the authors suggest that, 12 bp upstream of the previously identified heptamer in FR3, there is a novel nonamer consensus sequence, TCTGAG GAC, that serves as a specific signal for VH gene replacement in over 70% of VH genes (Chen et al., 1995b). It will be important to provide formal proof that this sequence actually functions as a nonamer.

More recent studies provide compelling evidence that the binding to dsDNA per se induces receptor editing (Chen et al., 1997). Analysis of hybridomas from double knock-in mice revealed that editing had occurred in a significantly greater percentage of B cells specific for dsDNA than in B cells that could only recognize ssDNA. Moreover, it was shown that B cells undergoing receptor

editing were much more likely to revise their knock-in L chain than to replace their knock-in H chain.

Receptor Editing and Repertoire Diversification

Although VL and VH gene replacements have been observed in AMuLV-transformed pre-B cell lines (see above), it is important that successive rearrangements are not restricted to transformed cells. During B cell proliferation, secondary rearrangements of κ chain alleles take place and may contribute to the diversification of the immune repertoire that is available for recognition of foreign antigens (Caton, 1990). One possible reason for editing could be the formation of a structurally compromised pair of μ H chain and surrogate L chain (Keyna et al., 1995; Ye et al., 1996). However, even after the formation of an immunoglobulin surface receptor, a population of B cells continues secondary rearrangements. In vitro, culture of immature sIg$^+$ B cells from Eμ–bcl-2 mice showed continued expression of recombination-activating genes, RAG-1 and RAG-2, and rearrangement of L chain loci. This rearrangement occurred in an ordered fashion, whereby κ-chain$^+$sIg$^+$ cells could become λ-chain$^+$sIg$^+$ cells (Rolink et al., 1993).

More recent studies in mice immunized with nitrophenyl acetate coupled to chicken γ-globulin showed RAG-1 and RAG-2 protein expression in centrocytes of mature splenic germinal centers (Han et al., 1996). Because adjacent sections showed the coincident expression of B7-2, a marker of centrocytes, it was concluded that V(D)J recombination is induced in B cells after encounter with antigen. A companion paper strengthened this conclusion by providing a phenotypic characterization of B cells in lymph nodes and in culture (Hikida et al., 1996). In the future, it will be important to establish whether RAG reactivation in germinal centers is induced by random somatic mutations that alter BCR specificity or convert it to self-reactivity, and whether this form of receptor editing generates BCR that will successfully compete for antigen.

Further evidence that editing plays a role in shaping the antibody repertoire comes from two experimental systems where "nonautoreactive" VHDJH rearrangements were introduced by homologous recombination at the JH locus. In the first set of experiments, a complete rearranged VHDJH gene coding for the T15 antibody specific for pneumococcal C-polysaccharide was inserted into the DQ52–JH region (Taki et al., 1995). More than half of B cells in the spleen and peripheral blood of heterozygous mutant animals expressed the wild-type IgH allele. This expression occurred through inactivation of the inserted VH gene on the mutant chromosome by rearrangement of upstream DH or VH and DH genes into the VT_{15} gene. Two additional heptamer RSS found in FR1 of the T15 VH gene are used preferentially and result in VH "silencing rearrangements." These studies suggest that not all VH genes are likely candidates for VH replacement, although they may provide an intermediate substrate suitable for receptor editing.

In a second set of studies, the stretch of genomic DNA containing the JH segments was replaced with a VHDJH segment which, in combination with a λ chain, imparts binding to the hapten NP (Cascalho et al., 1996). Crossing these mice to mice unable to express IgH and κ chains generates approximately 20% of B cells that make antibodies with no anti-NP antibody specificity. In these animals, the majority of serum immunoglobulin derives from NP-negative B cells that have undergone secondary VH gene rearrangements, apparently by a mechanism analogous to that seen in 3H9 "knock-in" mice.

If, as the experiments with the knock-in autoreactive mouse model suggest (Chen et al., 1995b), V gene replacements are triggered as a means to rescue high affinity autoreactive B cells from programmed cell death, it may seem surprising that VH replacement occurs so frequently in the B cells of mice expressing anti-NP or anti-pneumococcus antibodies. However, the findings that a proportion of antibodies to the hapten NP (Zouali et al., 1987) or to the pneumococcal cell wall (Putterman et al., 1996) also react with DNA suggest that self-cross-reactivity might have been the trigger of the BCR revision events seen in the two knock-in experimental systems discussed above (Taki et al., 1995; Cascalho et al., 1996). Alternatively, these rearrangements may reflect selected or stochastic events that serve to diversify the expressed repertoire.

Editing and Autoimmunity

Since editing provides an additional pathway to B cell tolerance, it is important to consider its relevance to pathogenic autoimmunity. It is now recognized that autoimmune diseases are complex and that different molecular mechanisms, combined with different environmental factors, probably trigger the same symptoms in patients with different genetic susceptibilities (Zouali, 1994b). Among the various contributing defects, alterations in the pathways of normal B cell tolerance and development are currently the focus of much investigation. Several lines of indirect evidence suggest that B cells can by themselves exhibit an intrinsic defect and that there is no need of a T cell defect or a failure in tolerant T cell help to generate autoreactive B cells (reviewed by Huck and Zouali, 1996). The view that, in normal individuals, encounter of potentially harmful, high affinity, self-reactive B cells with autoantigens triggers receptor editing raises the issue of whether B cells of autoimmune patients also attempt to switch off their autoreactivity by generating a second BCR through secondary L chain rearrangements (Figure 2). It has recently been noticed that human lupus anti-DNA antibodies representative of the pathogenic subset use essentially Vκ genes that are proximal to the Jκ cluster and that, in addition, they show a tendency to utilize upstream Jκ genes, suggesting that the corresponding B cells may be blocked in their capacity to undergo successive rearrangements and to edit their receptors (Bensimon et al., 1994). It is also of interest that in MRL/*lpr* autoimmune mice, the severity of the disease correlates with the biased use of the most distal J558 VH gene family—an observation that could be explained if VH replacements were more frequent in this experimental autoimmune model (Komisar et al., 1989). Both observations suggest that receptor editing must be a carefully controlled process in normal B cell development.

Table 1. A Heptamer-Like Sequence Is Embedded within the 3′ End of Human VH Genes

VH Gene Family	Number of Genes Positive/ Number of Genes Tested	Percent
VH1	11 of 11	100
VH2	3 of 3	100
VH3	18 of 22	87
VH4	11 of 11	100
VH5	2 of 2	100
VH6	1 of 1	100
VH7	1 of 1	100
Total	47 of 51	92

The heptamer-like sequence TACTGTG was found to be present in 51 human sequences representing essentially all known germline genes (reviewed by Zouali, 1994a; Cook and Tomlinson, 1995). Sequences were from the EMBL/GenBank data base.

Receptor Editing in Human B Cells

The possibility that human B cells can change their BCR has been addressed in several studies. Experiments in B cell lines suggest that secondary rearrangements can occur in the λ and κ chain loci (Combriato et al., 1991; Huber et al., 1992). The conclusion that the recombination machinery is not turned off by a productive rearrangement is supported by more recent studies of normal B cells. When sIg^+ immature B cells isolated from normal bone marrow were sorted into κ^+sIg^+ and λ^+sIg^+ cells, these sorted immature cells still expressed RAG-1. After 3 days of culture, 11%–24% of κ^+sIg^+ cells became λ^+sIg^+ cells. However, human peripheral B cells showed no changes in L chain phenotype, and RAG-1 was undetectable (Ghia et al., 1995). The changes in L chain expression of the $sIgM^+$ bone marrow cells suggest that surface expression of IgM does not terminate L chain rearrangements, but that the capacity for continued rearrangements is lost when B cells mature.

Analysis of the H chain locus in a B lymphoma cell line from a leukemia patient suggested that VH replacement occurs in vivo (Deane and Norton, 1990). The H chain locus of this patient showed a composite VH–VH–D–JH gene in which the upstream 5–51 VH gene, previously called VH251, was joined to the downstream 3–23, previously called VH26. VH replacement was further described in Epstein–Barr virus–transformed human B cell lines and in peripheral blood lymphocytes of healthy donors (Brokaw et al., 1992).

Since studies on secondary VH gene rearrangement in mouse B cells have shown that they are dependent on the presence of internal recombination signals (Kleinfield et al., 1986; Reth et al., 1986; Covey et al., 1990) and that heptamer-like sequences are common in the 3′ end of murine VH genes (Chen et al., 1995a), it was of interest to probe human VH genes for the presence of signal sequences. Examination of 51 human germline VH genes representing the seven known gene families (reviewed by Zouali, 1994a; Cook and Tomlinson, 1995) showed that a VH heptamer-like element lies in the 3′ end of virtually all known human VH genes (Table 1). Thus, the presence of the heptamer-like sequence in the 3′ end of human VH genes and the occurrence of VH gene replacement in untransformed human B lymphocytes (Brokaw et al., 1992) suggest that this mechanism may play a role in human antibody diversification.

Implications

In summary, ongoing successive rearrangements may be a more general phenomenon than had previously been assumed. It is important to emphasize that not all secondary rearrangements are coupled to editing and that some may be independent of antigen encounter. In addition to expanding antibody diversity, variable gene replacement may represent a mechanism that enhances the probability of a B cell viability if the primary rearrangement has resulted in a truncated variable gene segment or an autoreactive BCR. The implications of the discovery of receptor editing are numerous and exciting, and, naturally, a number of questions arise. When does editing stop, and can it be reactivated? What mechanism regulates receptor editing, and what gene products are involved? Is there an absolute requirement for RAG-1 and RAG-2, and are there other more specialized factors that facilitate editing? A major focus, then, becomes to delineate the timing of editing and its component steps. If variable gene replacement is triggered by engagement of the BCR with antigen, it should start at the immature IgM^+ B cell stage, after L chain assembly, as suggested by studies of B cell development by transgenic complementation of RAG-1- or RAG-2-deficient mice. In RAG-1- and RAG-2-deficient mice expressing an H chain transgene, differentiation in the bone marrow is arrested at the pre-B cell stage (Melchers et al., 1995; Spanopoulou et al., 1994; Young et al., 1994). By contrast, simultaneous introduction of μ H chain and λ L chain transgenes into $RAG\text{-}2^{-/-}$ mice leads to the generation of a substantial population of monoclonal B cells (Young et al., 1994), presumably incapable of editing. If, as seems likely, V gene replacement occurs in normal B cells, it will be important to see whether a recombinase activity remains present at low levels in B cells after functional variable gene assembly or whether this activity is triggered by specific stimuli, such as autoantigens. The experiments involving transgenic complementation (Spanopoulou et al., 1994; Young et al., 1994) and those using autoantibody transgenes (Tiegs et al., 1993; Radic et al., 1993a) suggest that RAG expression and successive rearrangements are triggered by ligation of the BCR on B cells. It is therefore conceivable that self-antigen stimulation of B cells represents the basis for the reactivation of the recombinase in normal B cells.

It remains, however, an intriguing possibility that editing is a constitutive property of immature B cells. With B cell maturation, editing ceases because further secondary rearrangements are prevented either by a decrease of RAG-1 and RAG-2 activity or by a change in the accessibility of the locus to the recombinases. Equally important will be to decipher the molecular mechanisms involved in editing. This will be possible by separating apoptotic B cells by FACS and examining their V genes (Maybaum and Reynolds, 1996). In the "knock-in" experiments described above (Chen et al., 1995b; Taki et al., 1995; Cascalho et al., 1996), there are a number of N sequences at the V–D–J junctions of variable genes rearranged as a result of editing. Since the enzyme TdT,

which mediates N sequence addition, is in principle only active at the pro-B cell stage, it may be that VH gene replacement occurs at this B cell developmental stage, i.e., before antigen exposure. Another possibility is that, as already proposed (Chen et al., 1995b), TdT is reactivated at the time of expression of *RAG* genes, which are mandatory for V–D–J gene assembly and editing. Also intriguing is whether and how receptor editing and clonal deletion operate in concert to maintain B cell tolerance. At present, the complex machinery responsible for deletion by programmed B cell death is the focus of much attention, with a number of genes and pathways identified (Rothstein, 1996). From recent studies, it appears that two genes, *Fas* and *bcl-x*, are not necessary for editing (Fulcher et al., 1996; Rubio et al., 1996). Finally, the role of the molecular cascade involved in transducing signals through the BCR has not been addressed. One of them, the B cell Src family kinase Lyn, seems to play an important role in the elimination of autoreactive B cells (Hibbs et al., 1995; Nishizumi et al., 1995; Huck and Zouali, 1997). Clearly, much more work is required to probe the role of BCR signaling and apoptosis-related genes in the regulation of receptor editing and to delineate the precise stages involved in this novel pathway.

Acknowledgments

M. Z. R. is an Arthritis Foundation Investigator and is supported by the National Institutes of Health (grant AI-34881) and an allocation by the Allegheny Singer Research Institute. M. Z. is an Investigator of the Institut National de la Recherche et de la Santé Médicale (INSERM). He is supported by grants from the Institut Pasteur and the Fondation pour la Recherche Médicale.

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